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(54) Title: CHEMOKINES

(57) Abstract: The interferon (IFN)-inducible chemokines, i.e. IFN-γ inducible protein-10 (IP-10), monokine induced by IFN-γ (Mig) and IFN-inducible T-cell α-chemoattractant (I-TAC), share a unique CXC chemokine receptor (CXCR3). Recently, a highly specific membrane-bound protease and lymphocyte surface marker CD26/dipeptidyl peptidase IV (DPP IV) was found to be responsible for posttranslational processing of chemokines. Removal of $NH_2$-terminal dipeptides by CD26/DPP IV alters chemokine receptor binding and signaling, and hence inflammatory and anti-HIV activities. CD26/DPP IV and CXCR3 are both markers for Th1 lymphocytes and, moreover, CD26/DPP IV is present in a soluble, active form in human plasma. Here we report that I-TAC was efficiently cleaved by CD26/DPP IV, whereas for Mig the kinetics were 10-fold slower. Processing of IP-10 and I-TAC by CD26/DPP IV resulted in reduced CXCR3 binding properties, loss of calcium signaling capacity through CXCR3 and more than 10-fold reduced chemotactic potency. Moreover, CD26/DPP IV-cleaved IP-10 acted as a chemotaxis antagonist. In contrast, CD26//DPP IV-truncated IP-10 retained its ability to inhibit the angiogenic activity of interleukin-8 in the rabbit cornea micropocket model. Our data demonstrate a negative feedback regulation by CD26/DPP IV in CXCR3-mediated inflammation without affecting the angiostatic potential of the CXCR3 ligand IP-10.



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# CHEMOKINES

## FIELD OF THE INVENTION

The invention relates to truncated chemokines as well as to a method to produce truncated chemokines, which have an anti-angiogenic effect while exercising a negative action on CXCR3-mediated inflammation.

## BACKGROUND OF THE INVENTION

Chemokines constitute a family of low molecular mass proteins that regulate the directed migration of specific subclasses of leukocytes during normal and inflammatory processes (1-3). The cellular specificity of chemokines is determined by the restricted expression of chemokine receptors on various leukocyte cell types (4). Chemokines are divided in subfamilies depending on the position of the first two cysteines in their primary sequence. The CC subfamily, with two adjacent cysteines, contains more than 20 different proteins that regulate the migration of monocytes, eosinophils, basophils, B and T lymphocytes, natural killer (NK) cells and dendritic cells. The CXC chemokine subfamily, with two cysteines separated by one other amino acid, contains several proteins with a Glu-Leu-Arg (ELR) motif in front of the first cysteine. These ELRCXC chemokines all attract neutrophilic granulocytes to sites of inflammation. The CXC chemokines without ELR-motif can attract monocytes, B and/or T lymphocytes. Three of the known non-ELR-CXC chemokines, i.e. the interferon-γ (IFN-γ) inducible protein-10 (IP-10 or CXCL10), monokine induced by IFN-γ (Mig or CXCL9) and IFN-inducible T-cell α-chemoattractant (I-TAC or CXCL11) recognize a single CXC chemokine receptor (CXCR), namely CXCR3 (5-8). IP-10, Mig and I-TAC attract monocytes and activated memory Th1, but not Th2, lymphocytes (9-11). Furthermore, eosinophils and subclasses of B and NK cells have been reported to express CXCR3 (11-12). In addition to their role in leukocyte migration, chemokines play a role in angiogenesis (13-16). ELRCXC chemokines have been reported to be angiogenic due to their interaction with CXCR2 (17-18). In contrast, the non-ELR-CXC chemokines IP-10, Mig and PF-4 (platelet factor-4 or CXCL4) were found to have angiostatic activity but the molecular mechanism is not understood (15).

The $NH_2$-terminal region of most chemokines is crucial for receptor binding and signaling activities. Some chemokines become chemotactic only when processed at the $NH_2$-terminus, e.g. truncation of platelet basic protein (PBP) into NAP-2 (CXCL7) (19). Others, e.g. the monocyte chemotactic proteins MCP-1 (CCL2), MCP-2 (CCL8) and MCP-3 (CCL7), loose their chemotactic activity when $NH_2$-terminal amino acids are cleaved off (20,21). Aminopeptidases or endopeptidases that process chemokines at the $NH_2$-terminus play an important role in the up- or down-regulation of chemokine activities. One of these enzymes, the membrane-associated protease dipeptidyl peptidase IV (DPP IV, EC3.4.14.5) is highly specific. It cleaves off dipeptides from polypeptides with a proline, alanine or hydroxyproline at the second position. DPP IV, which is expressed on fibroblasts, epithelial and endothelial cells, is identical to the lymphocyte surface glycoprotein and T-cell activation marker CD26 (22). The extracellular domain of CD26/DPP IV also exists as a soluble and proteolytically active form in plasma and in cerebrospinal and seminal fluids. CD26/DPP IV interacts with CD45 (a protein tyrosine phosphatase) and with adenosine deaminase, has costimulatory activity in T-cell immune responses, plays a role in immune processes such as allograft rejection, suppresses malignant transformation and has been implicated in the regulation of insulin secretion (22,23). Recently, CD26/DPP IV was found to cleave a number of chemokines, but not cytokines (24). Moreover, one of the chemokine receptors that has been identified as a Th1 cell marker, in addition to CCR5, is CXCR3 (9-11). Here we report that CD26/DPP IV efficiently cleaved all three CXCR3 ligands and demonstrate that the protease differently effects the receptor signaling, lymphocyte chemotactic and anti-angiogenic activities of these chemokines.

## SUMMARY OF THE INVENTION

This invention involves rapid enzymatic processing of three interferon-induced CXC chemokines that bind to the same receptor and Th1 lymphocyte marker CXCR3. The clipping enzyme is another Th1 marker CD26/dipeptidyl peptidase IV (DPP IV). The reduction in signaling and chemotactic potency of the chemokines IP-10, Mig and I-TAC, after proteolytic processing by CD26/DPP IV, is demonstrative for a natural negative feedback mechanism in the inflammatory response. This includes inactivation of the CXCR3 ligands as chemoattractants, whilst retaining competition with intact chemokine for receptor binding. In contrast, the anti-angiogenic potential of IP-10 remains unaffected by

the clipping. This indicates that the molecular mechanisms of action that underlie the inflammatory and anti-angiogenic activities of these chemokines are different and that the angiostatic activity does not necessarily imply an activation of the IP-10 receptor CXCR3.

Therefore, a first object of this invention is a method of truncating CXC chemokines, characterised in that said chemokines are exposed to or treated with a lymphocyte membrane protease, or variants, homologues or fragments of said lymphocyte membrane protease and said truncated chemokines have reduced CXCR3 binding properties, loss of calcium signalling capacity through CXCR3, a reduced chemotactic potency and said truncated chemokines have retained ability to inhibit angiogenic activity of cells. In a preferred embodiment the method comprises enzymatic clipping of the CXC chemokines using the protease CD26/dipeptidyl peptidase IV or variants, homologues or fragments thereof. Preferably, said truncated CXC chemokines are derived from the chemokines selected from the group consisting of interferon (IFN)-inducible chemokines, monokine induced by IFN-γ (Mig) and IFN-inducible T-cell α-chemoattractant (I-TAC). More preferably, said truncated CXC chemokines are derived from the chemokine IFN-γ inducible protein-10 (IP-10).

A second object of the invention are the truncated CXC chemokines or their variants, homologues or fragments having reduced CXCR3 binding properties, loss of calcium signalling capacity through CXCR3 and reduced chemotactic potency and said truncated chemokines having retained ability to inhibit angiogenic activity of cells, said chemokines being obtainable by said method.

Truncation of the CXC chemokines according to said method has the advantage that it results in an inactivation of the CXCR3 ligands as chemoattractants, whilst retaining competition with intact chemokine for receptor binding. In consequence, the said truncated CXC chemokines have an inhibitory action on CXCR3-mediated inflammation. In contrast, the anti-angiogenic potential of CXC chemokines remains unaffected by the clipping. Therefore, a third object of this invention is the use of the truncated CXC chemokines for the treatment of inflammatory and angiogenesis-dependent diseases, such as rheumatoid arthritis, restenosis, cancer, multiple sclerosis and psoriaris.

## ILLUSTRATIVE EMBODIMENT OF THE INVENTION

### Legends to the figures

**Figure 1: Time course of the truncation of CXCR-3 ligands by CD26/DPP IV**

IP-10 (diamonds), Mig (circles) and I-TAC (triangles) at 5 μM were incubated for different time intervals with 25 U/l soluble CD26/DPP IV as indicated in the Methods section. The remaining concentration of the intact CXCR3-ligands determined by mass spectrometry is indicated in the graphs.

**Figure 2: Effect of CD26/DPP IV on the lymphocyte chemotactic activity of IP-10**

The chemotactic activity of intact (filled diamonds) and CD26/DPP IV-truncated (open diamonds) IP-10 for PHA- (panel A) or anti-CD3-stimulated (panel B) lymphocytes was measured in the Boyden chamber assay. Results represent the mean (± SEM) chemotactic index of 4 or more independent experiments with cells from different donors. The Student t-test was used for statistical analysis (** = $p<0.05$; *** = $p<0.01$ for a significantly positive chemotactic response compared to buffer controls).

**Figure 3: Comparison of the chemotactic activity and calcium mobilizing capacity of intact and truncated CXCR3 ligands on CXCR3-transfected cells.**

Intact (filled symbols) and CD26/DPP IV-truncated (open symbols) IP-10 (diamonds), Mig (circles) and I-TAC (triangles) were tested for their ability to induce a chemotactic response or to increase the $[Ca^{2+}]_i$ in CXCR3-transfected CHO cells. Results represent the mean (± SEM) chemotactic index of 4 or more independent experiments and the mean (± SEM) increase of the $[Ca^{2+}]_i$ of 5 or more independent experiments. The detection limit for the increase of the $[Ca^{2+}]_i$ is indicated by the dashed line. The Student t-test was used for statistical analysis (* = $p < 0.1$; ** = $p<0.05$; *** = $p<0.01$ for a positive chemotactic response compared to buffer controls).

**Figure 4: Receptor binding properties of intact and CD26/DPP IV-truncated CXCR3 agonists**

Intact (filled symbols) and CD26/DPP IV-truncated (open symbols) IP-10 (diamonds), I-TAC (triangles) and Mig (circles) were tested for their ability to compete for $^{125}$I-labeled

I-TAC binding to peripheral blood-derived mononuclear cells or CXCR3-transfected CHO cells. Results represent the mean (± SEM) % of $^{125}$I-I-TAC that binds to the cells compared to the amount of labeled I-TAC that binds the cells without addition of cold ligands (3 or more independent experiments). The Student t-test was used for statistical analysis (** = $p<0.05$; *** = $p<0.01$ for a significant difference between the intact and truncated chemokines).

**Figure 5: Inhibition of chemotaxis by truncated IP-10(3-77)**

The antagonistic activity of CD26/DPP IV-truncated IP-10 was tested on CXCR3-transfected cells in the Boyden microchamber. Intact IP-10 (at 10 or 30 nM) was added with (black histograms) or without (open histograms) 30 nM IP-10(3-77) in the bottom well of the chemotaxis chamber. Results represent the mean (± SEM) chemotactic index (C.I.) from 5 independent experiments. The Student t-test was used for statistical analysis (** = $p<0.05$; *** = $p<0.01$).

**Figure 6: Comparison of the anti-angiogenic activity of IP-10 and IP-10(3-77)**

Hydron pellets were implanted in a corneal micropocket on a rabbit eye. Hydron pellets contained dilution buffer (panel A), 3 ng of natural human IL-8 (panel B), 3 ng of natural human IL-8 and 10 ng of intact IP-10(1-77) (panel C), or 3 ng of natural human IL-8 and 10 ng of IP-10(3-77) (panel D). Neovascularization was scored daily (score from 0 to 4) from day 4 to 8 and the maximal neovascularization (always occurring between day 5 and 7) is shown in panel E (each dot represents an independent experiment). The mean values are indicated by the lines and statistical analysis (comparison with the positive control that contained 3 ng IL-8) was performed using the Mann-Whitney U-test. The photographs in panels A to D are representative examples for the mean neovascularization scores as indicated in panel E.

**Example 1:** General method examples

*1a) Reagents and cell lines*

Recombinant human chemokines (IP-10, Mig and I-TAC) and recombinant human IL-2 were purchased from PeproTech (Rocky Hill, NJ). Natural IL-8 was purified to homogeneity from conditioned medium from monocytes and contained an equimolar mixture of IL-8(1-77), IL-8(5-77) and IL-8(6-77) (19). Soluble natural human CD26/DPP IV, without membrane anchor and starting at amino acid Gly31, was obtained from total seminal plasma and purified to homogeneity by anion exchange followed by affinity chromatography on immobilized adenosine deaminase as described (28). Human CXCR3 was stably expressed in CHO-K1 cells (ATCC: CCL-61) and cultured in HAM'S F-12 medium (Biowhittaker Europe, Verviers, Belgium) supplemented with 10 % FBS, 1 mM sodium pyruvate and 400 µg/ml geneticin.

*1b) In vitro truncation of chemokines by CD26/DPP IV*

In order to obtain efficient truncation, chemokines were treated with soluble CD26/DPP IV for 18 h at 37 °C and were subjected to SDS-PAGE as previously described (29). $NH_2$-terminal truncation was verified by Edman degradation on a pulsed liquid phase 477A/120A protein sequencer after electroblotting 0.5 to 2 µg of processed chemokine from the gel to Problot membranes (Applied Biosystems, Foster City, CA). Alternatively, truncated chemokines were purified by C8 RP-HPLC on a Brownlee Aquapore RP-300 column (50 x 1 mm; PerkinElmer, Norwalk, CT) and the average $M_r$ was determined by electrospray ion trap mass spectrometry (Esquire-LC; Bruker Daltonic, Bremen, Germany). To determine the time-course of the $NH_2$-terminal truncation, IP-10, Mig and I-TAC (5 µM) were incubated with soluble CD26/DPP IV (250, 25, 2.5 and 0.25 U/l) in 15 µl of 50 mM Tris buffer, pH 7.5, supplemented with 1 mM EDTA. The specificity of the reaction was checked by incubating the chemokines with Tris buffer alone. Samples (5 µl) were withdrawn after 5, 15 and 30 min, and the reaction was stopped by addition of trifluoroacetic acid (final concentration of 0.1 %). The samples were desalted on a C18 ZipTip (Millipore, Bedford, MA) and the relative amounts of the $NH_2$-terminally truncated chemokines were determined by ion trap mass spectrometry. The time course of the truncation (shown in the figures) was constructed by normalizing the incubation times,

taken into account that 25 U/l is close to the normal serum concentrations of DPP IV (30).

*1c) Chemotaxis assays*

Peripheral blood mononuclear cells were purified from buffy coats from healthy volunteers as previously described (31). Mononuclear cells were stimulated with anti-CD3 antibodies (OKT3: ATCC CRL 8001) in RPMI 1640 (Biowhittaker Europe) with 10 % FBS for 2 days before use. Alternatively, mononuclear cells were cultured in RPMI 1640 with 10 % FBS and treated with PHA (2 µg/ml) for 3 days, washed with RPMI 1640 and kept in culture for two to three weeks in RPMI 1640 supplemented with 10 % FBS and 50 U/ml IL-2 before use in the chemotaxis assay (7).

Lymphocyte chemotaxis was performed in Boyden microchambers (Neuro Probe, Cabin John, MD) with fibronectin-coated, polyvinylpyrrolidone-free polycarbonate membranes (5 µm pore size, Corning Separations Division, Acton, MA). Lymphocytes were suspended in HBSS + 0.5 % (v/v) human serum albumin (HSA) at $2x10^6$ cells/ml and were allowed to migrate for 2 h at 37 °C. Before chemotaxis, CXCR3 transfected CHO cells were resuspended in HBSS + 0.5 % HSA and diluted to $1.5x10^6$ cells/ml. Boyden chamber chemotaxis experiments were performed for 2 h at 37 °C with 8 µm pore size polyvinylpyrrolidone-free polycarbonate membranes. In order to study antagonization, truncated chemokines were added at inactive concentrations together with the active substance to the bottom well of the Boyden chambers. Cells that migrated through the membrane were stained with DiffQuick (Merck, Darmstadt, Germany) and counted microscopically in 10 oil immersion fields (500 x magnification). The chemotactic index was calculated as the number of cells migrated to the sample divided by the number of cells spontaneously migrated to the sample dilution medium (HBSS + 0.5 % HSA).

*1d) Calcium signaling and receptor binding assays*

Alterations in intracellular calcium concentration ($[Ca^{2+}]_i$) in response to chemokines were monitored by fluorescence spectrometry. Briefly, CXCR3 transfected cells were loaded with the fluorescent dye Fura-2-AM for 30 min at 37 °C as previously described (29). Cells were washed with buffer, kept at 4 °C and preincubated for 10 min at 37 °C before use. $[Ca^{2+}]_i$ were measured in an LS50B spectrofluorimeter (PerkinElmer) at a final cell concentration of $10^6$/ml.

Competition for $^{125}$I-labeled I-TAC binding was measured on freshly isolated peripheral blood mononuclear cells or on CXCR3-transfected cells as described (32). Briefly, 2 x $10^6$ cells were incubated for 2 h at 4 °C with 0.06 nM $^{125}$I I-TAC (Amersham Pharmacia Biotech, Uppsala, Sweden) and varying concentrations of unlabeled chemokine. Cells were centrifuged and washed three times with 2 ml of PBS supplemented with 2 % (w/v) BSA and the radioactivity present on the cells was measured in a gamma counter.

*1e) In vivo test for anti-angiogenic activity of chemokines*

Chemokines were tested for their angiogenic or angiostatic activity in the rabbit cornea micropocket model (16). Briefly, 32 mg sucralfate (Merck) were dissolved in 72 µl PBS. Four µl of this sucralfate solution was mixed with 4 µl Hydron solution (12 % Hydron in ethanol; Interferon Sciences, New Brunswick, NJ) and 5 µl pellets were allowed to dry under UV light for 20 minutes. Subsequently, different concentrations of chemokines or dilution buffer (negative control) were dried on the pellets. One pellet was implanted 1 mm from the limbus into a corneal micropocket of each eye of an anesthetized New Zealand White rabbit. Neovascularization of the cornea was scored daily from day 5 to day 8 after implantation of the pellet. Maximal neovascularization was obtained between day 5 and 7. This maximal neovascularization was used for comparison.

**Example 2:** *Processing of CXCR3 ligands by CD26/DPP IV*

Incubation of IP-10, I-TAC and Mig with CD26/DPP IV resulted in the effective removal (> 95 %) of the $NH_2$-terminal dipeptide (Table 1). After 18 h of incubation with CD26/DPP IV, no remaining intact chemokine was detectable by Edman degradation. Proteolysis beyond the penultimate proline was not observed confirming the purity and specificity of CD26/DPP IV. For biological evaluation, proteolytically cleaved CXCR3-ligands were purified by C-8 RP-HPLC. The purity and $M_r$ of the cleaved chemokines were confirmed by mass spectrometry, which excludes carboxyterminal or internal processing (Table 1).

Mass spectrometry was used to study the time course of chemokine processing (Fig. 1). When 5 µM IP-10 was incubated with serum concentrations (25 U/l) of CD26/DPP IV, 50 % of the chemokine was truncated within the first 5 min and after 20 min less than 10 % of

the IP-10 proteins remained intact. In contrast, the kinetics of Mig processing were about 4 times slower (50 % conversion in 20 min) than that of IP-10, whereas 50 % and 90 % of intact I-TAC was converted into I-TAC(3-73) within 1.5 and 6 min, respectively. Thus, CD26/DPP IV processed I-TAC more than 10-fold faster than Mig.

***Example 3:*** *Impaired lymphocyte chemotactic activity of IP-10 after CD26/DPP IV cleavage*

The chemotactic activity of intact and CD26/DPP IV-truncated IP-10 was compared on activated (by PHA or anti-CD3) lymphocytes. On PHA-stimulated lymphocytes (Fig. 2A), a dose-dependent chemotactic effect was obtained with intact IP-10 from 1 nM onward, whereas IP-10(3-77) was still inactive at concentrations as high as 10 nM. Thus, processing of IP-10 by CD26/DPP IV resulted in a 30-fold reduction in lymphocyte chemotactic activity. Similar results were observed with anti-CD3-activated lymphocytes (Fig. 2B), although these cells were less sensitive to IP-10-induced chemotaxis than PHA-stimulated lymphocytes. This observation correlates with the reported lower expression of CXCR3 on anti-CD3-stimulated cells (5).

***Example 4:*** *Effect of CD26/DPP IV on the chemotactic and calcium signaling capacities of IP-10, Mig and I-TAC on CXCR3-transfected cells*

Only one receptor for IP-10, i.e. CXCR3, has been identified (6). Therefore, the chemotactic potencies of IP-10 and IP-10(3-77) were compared on CHO cells transfected with CXCR3 (Fig. 3). For IP-10, a dose-dependent (minimal effective concentration of 3 nM) chemotactic response was observed, whereas for CD26/DPP IV-truncated IP-10(3-77) the minimal effective concentration (100 nM) was 30-fold higher. Intact Mig and I-TAC induced a significant ($p<0.01$) chemotactic response on CXCR3-transfected cells at 10 nM. In contrast, truncated Mig(3-103) and I-TAC(3-73) were inactive at concentrations as high as 100 nM and 25 nM, respectively. Although their $NH_2$-terminal amino acid is different, removal of two amino acids (including the penultimate proline) resulted in a similarly impaired chemotactic activity of all three CXCR3 agonists.

Furthermore, it was observed that intact IP-10 and I-TAC induced a significant increase in $[Ca^{2+}]_i$ at concentrations higher than 1 nM, whereas 10-fold higher concentrations of Mig were required to obtain a detectable calcium response in CXCR3-transfected CHO cells (Fig. 3). Both IP-10 and I-TAC, truncated by CD26/DPP IV, lacked the calcium-signaling capacity through CXCR3. Indeed, although intact IP-10 induced calcium mobilization at 1 nM, IP-10(3-77) was inactive in the calcium assay at concentrations as high as 60 nM. In contrast, no significant reduction in the weak calcium mobilizing capacity of Mig through CXCR3 could be observed for Mig(3-103). In conclusion, although none of the three ligands possessed CXCR3-mediated chemotactic activity after $NH_2$-terminal truncation by CD26/DPP IV, Mig, but not IP-10 or I-TAC, retained its rather weak calcium signaling potency through CXCR3.

***Example 6:*** *Binding properties of intact and CD26/DPP IV-truncated CXCR3-ligands*

The capacity of truncated IP-10 to compete for binding of $^{125}$I-labeled intact I-TAC to peripheral blood-derived mononuclear cells was significantly decreased (Fig. 4). At 100 nM IP-10(3-77) displaced only 49 % of the labeled I-TAC from the cells, whereas the displacement with 100 nM intact IP-10 was 88 %. A comparable difference in binding competition capacity with $^{125}$I-labeled I-TAC to mononuclear cells was observed between intact and truncated I-TAC. On CXCR3-transfected cells, the binding affinity of IP-10(3-77) and I-TAC(3-73) was also clearly reduced (Fig. 4). No significant difference in binding competition capacity to mononuclear cells was observed between both Mig forms although there was a tendency for reduced binding potency for the truncated Mig. In addition, intact and truncated Mig could not significantly compete for $^{125}$I-I-TAC binding to CXCR3-transfected cells.

***Example 7:*** *Anti-inflammatory effect of truncated IP-10(3-77)*

Since IP-10(3-77) failed to induce calcium mobilization and chemotactic responses, but retained some receptor binding properties, this truncated chemokine was tested as an

inhibitor in chemotaxis assays on CXCR3-transfected cells. Addition of an inactive concentration IP-10(3-77) (30 nM) to the lower well of the Boyden chamber resulted in a more than 50 % reduction in chemotactic response towards 30 nM of intact IP-10 and a complete inhibition of the chemotactic response towards 10 nM intact IP-10 (Fig. 5).

***Example 8:*** *Anti-angiogenic activities of IP-10 processed by CD26/DPP IV*

When pellets containing 3 ng of natural human IL-8 were implanted into corneal micropockets on rabbit eyes, the induced neovascularization was maximal between day 5 and 7. Maximal inhibition of the angiogenic effect of IL-8 (3 ng) was obtained by addition of 10 ng of intact IP-10 (Fig. 6). Similarly, CD26-truncated IP-10(3-77) equally inhibited IL-8-induced angiogenesis. At 10 and 30 ng per pellet, no significant differences (Mann-Whitney U-test, $p > 0.2$) between the anti-angiogenic activities of intact and truncated IP-10 were observed. Although there was a tendency for intact IP-10 to be somewhat more efficient, these data indicate that in contrast to the inflammatory effect, the angiostatic potential of IP-10 is either not mediated through CXCR3 or implicates alternative CXCR3-triggered signal transduction pathways different from calcium signaling and chemotactic activity.

**Discussion**

During the past two decades, more than 50 human chemokines have been identified. Chemokines and chemokine receptors form a complex network that controls leukocyte migration during normal cell homing as well as inflammatory processes. The receptor specificity of chemokines determines the pattern of target cells. The expression of chemokines and chemokine receptors is regulated at the transcriptional level by different inducers. IFN-γ, a prototypic Th1 cytokine, has been reported to enhance the production of the non-ELR-CXC chemokines IP-10, Mig and I-TAC (7,33-36) but down-regulates the production of neutrophil chemotactic chemokines such as the ELRCXC chemokines IL-8 and ENA-78 (37). All three IFN-inducible non-ELR-CXC chemokines interact with CXCR3. This receptor is highly expressed on activated memory T cells (CD45RO+ cells)

and has been detected on a small portion of B cells and NK cells (5,11). Higher CXCR3 expression levels were detected on Th1 lymphocytes compared to Th2 cells and IP-10 has been reported to attract Th1 but not Th2 cells (9). Additional regulatory mechanisms of chemokine activity and receptor specificity were shown at the posttranscriptional level, mainly by $NH_2$-terminal chemokine processing. For example, limited $NH_2$-terminal truncation of IL-8 (e.g. by gelatinase B) enhanced its chemotactic potency whereas platelet basic protein was found to become chemotactic only when truncated into NAP-2 (19,38). In contrast, small modifications of the $NH_2$-terminal residues of MCP-1, MCP-2 and MCP-3 decreased the biological activity of these CC chemokines (20,21).

Recently, the highly specific protease CD26/DPP IV has been reported to $NH_2$-terminally process a number of chemokines with different effects on their chemotactic and antiviral activities (22,24). CD26 is expressed on a wide variety of cells including fibroblasts, epithelial and endothelial cells. Moreover, CD26 expression on CD45RO+ Th1 lymphocytes is further increased upon activation. This protease cleaves GCP-2, SDF-1, RANTES, eotaxin, macrophage-derived chemokine (MDC), and the macrophage inflammatory protein-1α (MIP-1α) isoform LD78β into $NH_2$-terminally truncated forms (22,24). Although MCP-1, MCP-2, MCP-3 and MIP-1β also possess the prerequisite penultimate proline, they are not processed by CD26/DPP IV. MCPs are protected from the proteolytic activity by their $NH_2$-terminal pyroglutamic acid (39). Here we report that CD26/DPP IV is able to efficiently cleave the $NH_2$-terminal dipeptide of all three CXCR3 ligands IP-10, Mig and I-TAC. Time-course experiments showed that under physiological serum concentrations of CD26/DPP IV, the majority of the chemokine is processed within a few minutes even when high concentrations of chemokine (5 μM) are present. This rapid processing of the CXCR3 ligands by CD26/DPP IV at physiological concentrations is indicative for an important functional role of this interaction between this ubiquitous protease and these chemokines.

The chemotactic potency of the CXC chemokine SDF-1 and of the CC chemokines RANTES, eotaxin and MDC was drastically reduced upon $NH_2$-terminal truncation by CD26/DPP IV (24). Accordingly, the binding affinity of truncated SDF-1, eotaxin and MDC for their respective receptors CXCR4, CCR3 and CCR4 decreased. In contrast, treatment of the CC-chemokine LD78β with CD26/DPP IV generated a highly potent monocyte and lymphocyte chemoattractant that retained strong anti-HIV-1 activity (40).

The limited $NH_2$-terminal processing of IP-10 by CD26/DPP IV described here resulted in 30-fold reduced lymphocyte chemotactic activity. All three truncated CXCR3 ligands, i.e. IP-10, Mig and I-TAC, lost chemotactic activity mediated through CXCR3. The calcium signaling capacity of IP-10 and I-TAC through CXCR3 was also abolished upon $NH_2$-terminal truncation. CD26/DPP IV truncated IP-10 and I-TAC retained weak CXCR3 binding properties, but higher doses of the truncated variant versus intact chemokine were necessary to remove $^{125}$I-I-TAC from its receptor. Finally, on CXCR3 transfected cells, truncated IP-10 acted as a chemotaxis inhibitor for the intact chemokine.

So far, no information was available on the role of CXCR3 in the angiostatic activity of IP-10. CD26/DPP IV-truncated IP-10 retained anti-angiogenic activity in the rabbit cornea micropocket model. Thus, CXCR3-mediated chemotactic activity and calcium signaling are no prerequisites for the angiostatic activity of IP-10. Since CD26/DPP IV and CXCR3 expression and IFN-γ production (the main inducer of the CXCR3 ligands) are hallmarks for activated memory Th1 cells, processing of the three chemokines by CD26/DPP IV may constitute an important physiological regulatory mechanism during the attraction of leukocytes in case of a typical Th1 response. Indeed in the case of a typical Th1 response, it is crucial to dampen the cellular reaction after sufficient cell influx has occurred. Chemo-attracted Th1 lymphocytes, laden with CD26/DPP IV, cleave the angiostatic chemokines IP-10, Mig and I-TAC resulting in down-modulation of their chemotactic activity. If the angiostatic effects would also be reduced, then the balance would be in favor of angiogenesis which may bring the local vessels and the circulating leukocytes closer to the Th1 response site. Obviously, the latter effect does not take place and angiostasis thus helps to keep the circulating lymphocytes out of the field of attraction and to halt the inflammation. As a consequence, the interaction between CD26/DPP IV and CXCR3-ligands may play an important role in disease processes such as multiple sclerosis (41-43), B-cell leukemia (44), hepatocellular carcinoma (45), and skin diseases (34,25-26) where expression of CXCR3 and its ligands have been reported.

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**Tables**

**Table 1: $NH_2$-terminal sequence analysis and mass spectrometry of CD26/DPP IV-treated chemokines.**

| Chemokine | $NH_2$-terminal sequence[A] | | Reduction in $M_r$ | |
|---|---|---|---|---|
| | Untreated | CD26/DPP IV treated | theoretical | observed[B] |
| IP-10 | VPLSRTVRCTC | LSRTVRCTC | 196.25 | 196.9 ± 0.4 |
| Mig | TPVVRKGRCSC | VVRKGRCSC | 198.22 | 197.7 ± 0.4 |
| I-TAC | FPMFKRGRCLC | MFKRGRCLC | 244.29 | 243.9 ± 0.3 |

[A] The $NH_2$-terminal sequence of untreated and CD26/DPP IV treated (18 h incubation) chemokines was determined by automated Edman degradation.

[B] The average (± SD) difference between the $M_r$ of intact and truncated CXCR3 ligands was calculated from mass measurements on 4 different incubations (between 2 and 30 min) with CD26/DPP IV.

CLAIMS

1. A method of truncating chemokines, characterised in that said chemokines are exposed to or treated with a lymphocyte membrane protease, or variants, homologues or fragments of said lymphocyte membrane protease and said truncated chemokines have reduced CXCR3 binding properties, loss of calcium signalling capacity through CXCR3, a reduced chemotactic potency and said truncated chemokines have retained ability to inhibit angiogenic activity of cells.

2. The method of claim 1, characterised in that said lymphocyte membrane protease is a CD26/dipeptidyl peptidase IV or variants, homologues or fragments thereof.

3. The method of claim 2, characterised in that the chemokines were treated at least during 5 hours and at least at 15 °C with CD26/dipeptidyl peptidase IV or to variants, homologues or fragments thereof.

4. The method of claim 1 to 3, characterised in that said chemokines are CXC chemokines.

5. The method of claim 1 to 3, characterised in that the chemokines are selected from the group consisting of interferon (IFN)-inducible chemokines, monokine induced by IFN-γ (Mig) and IFN-inducible T-cell α-chemoattractant (I-TAC).

6. The method of claim 1 to 3, characterised in that the chemokine is IFN-γ inducible protein-10 (IP-10).

7. Truncated CXC chemokines or their variants, homologues or fragments having reduced CXCR3 binding properties, loss of calcium signalling capacity through CXCR3 and reduced chemotactic potency and said truncated chemokines having retained ability to inhibit angiogenic activity of cells, said chemokines being obtainable by any of the processing method of claim 1 to 6, said processed chemokine.

8. The CXC chemokines of claim 7 for use in a treatment of multiple sclerosis.

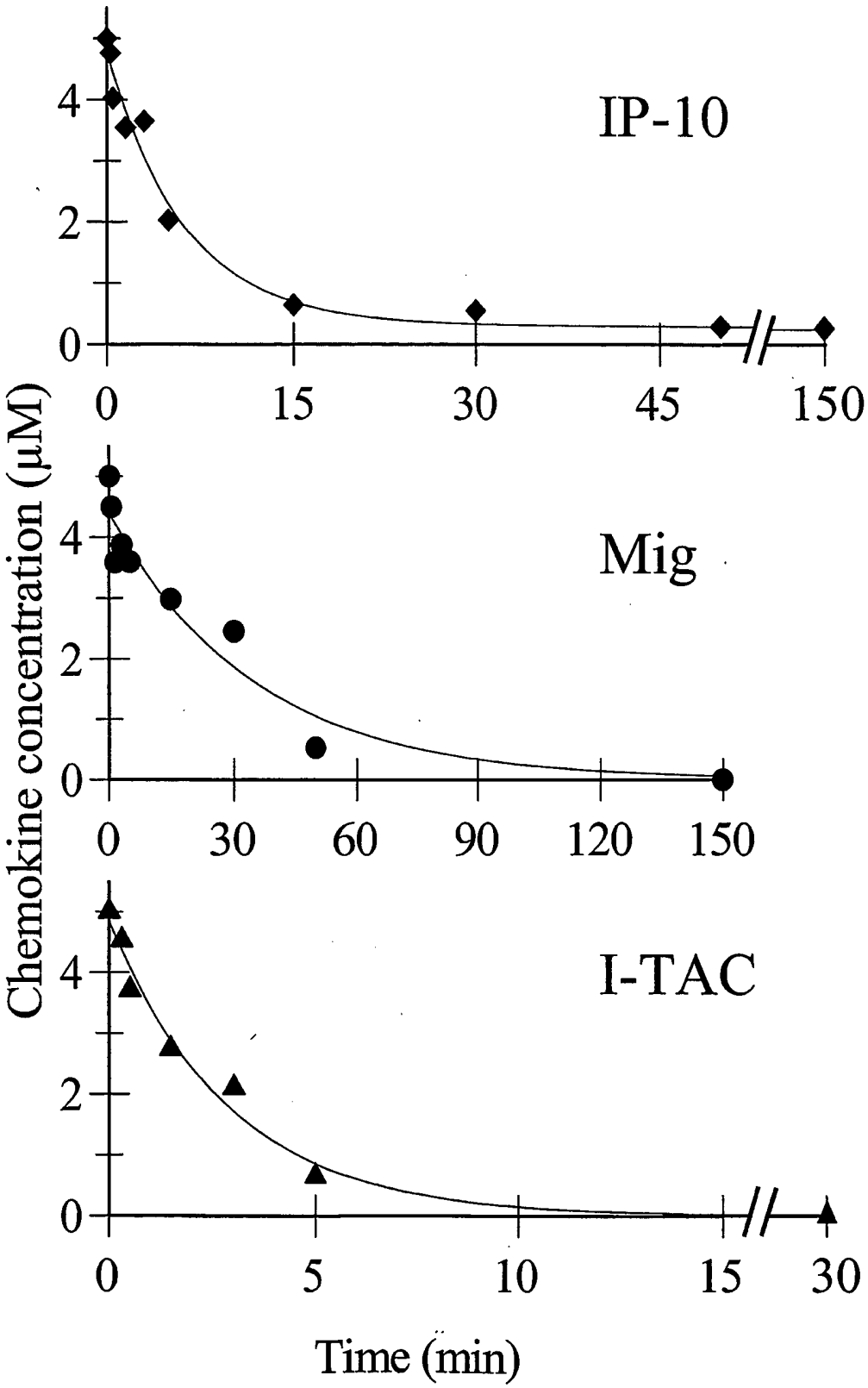
9. The CXC chemokines of claim 7 for use in a treatment of rheumatoid arthritis.

10. The CXC chemokines of claim 7 for use in treatment of cancer.

11. The CXC chemokines of claim 7 for use in a treatment of B-cell leukemia.

12. The CXC chemokines of claim 7 for use in a treatment of hepatocellular carcinoma.

13. The CXC chimokines of claim 7 for use in a treatment of skin diseases.


IP-10
Mig
I-TAC
Chemokine concentration (µM)
Time (min)
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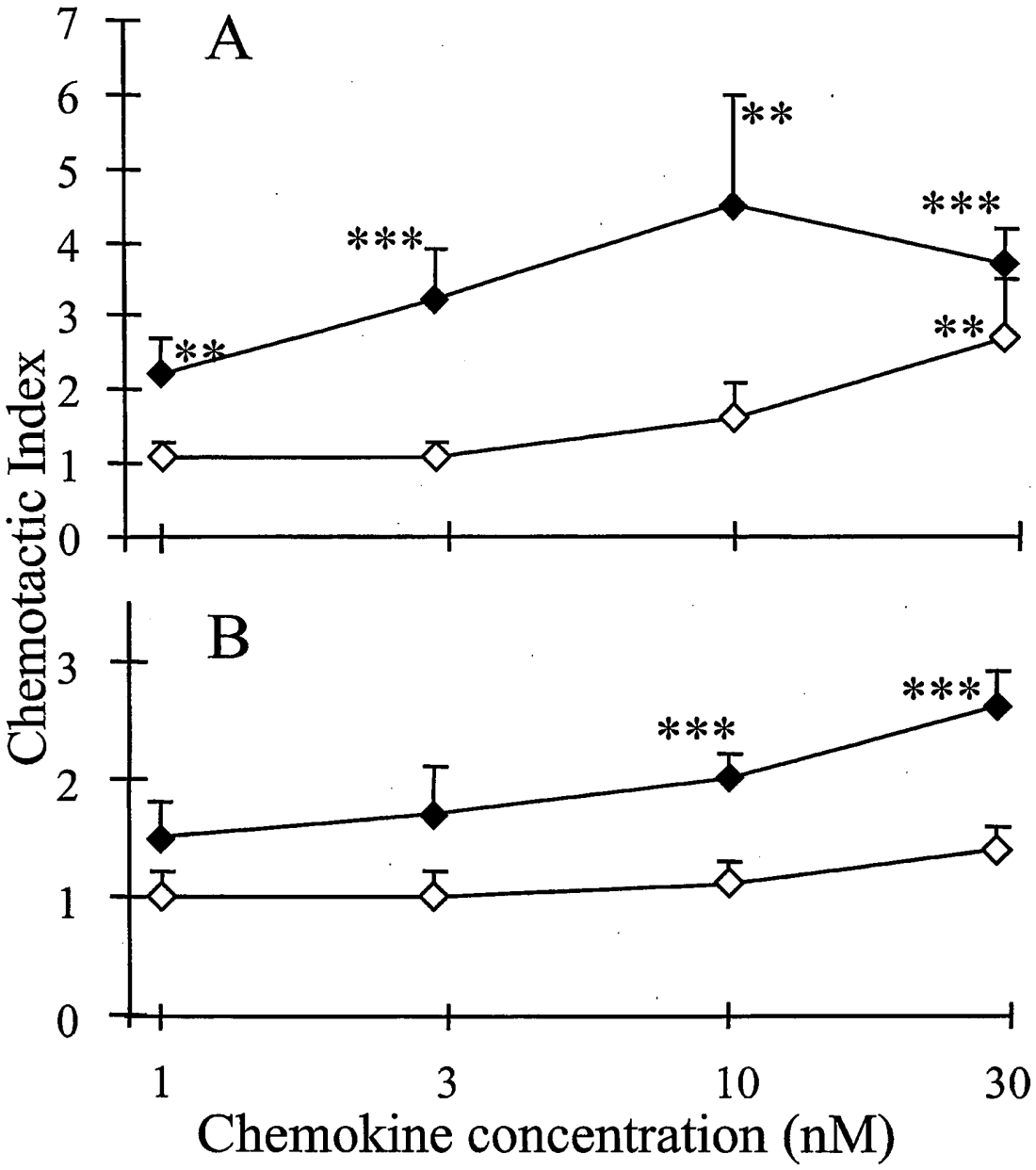
0
15
30
45
150
0
30
60
90
120
150
0
5
10
15
30


A
B
Chemotactic Index
Chemokine concentration (nM)
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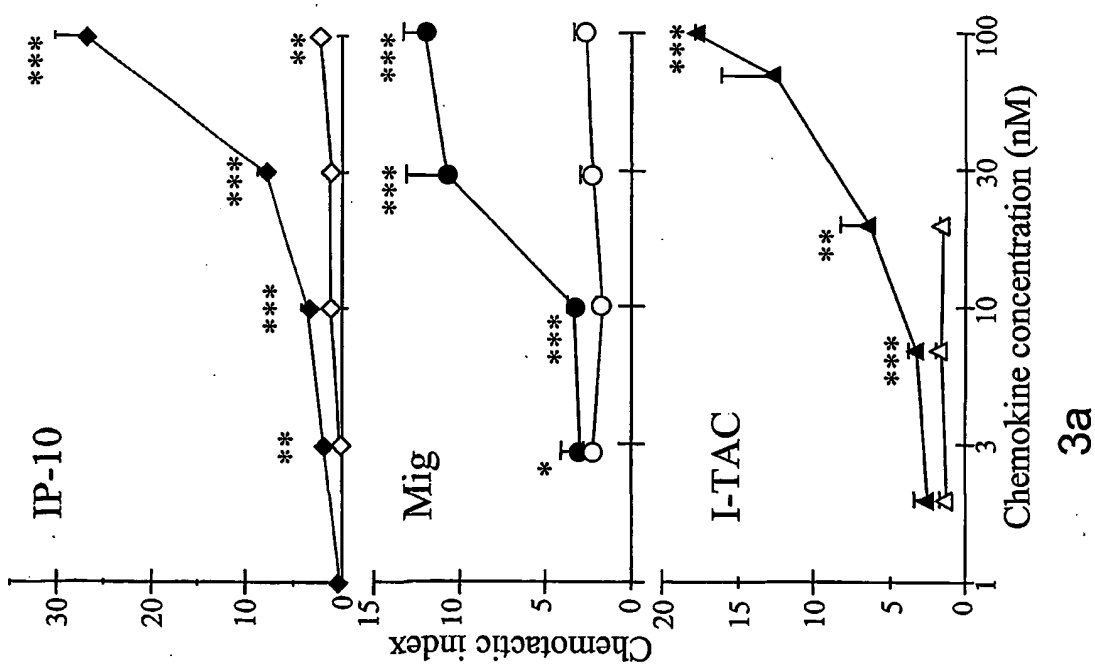
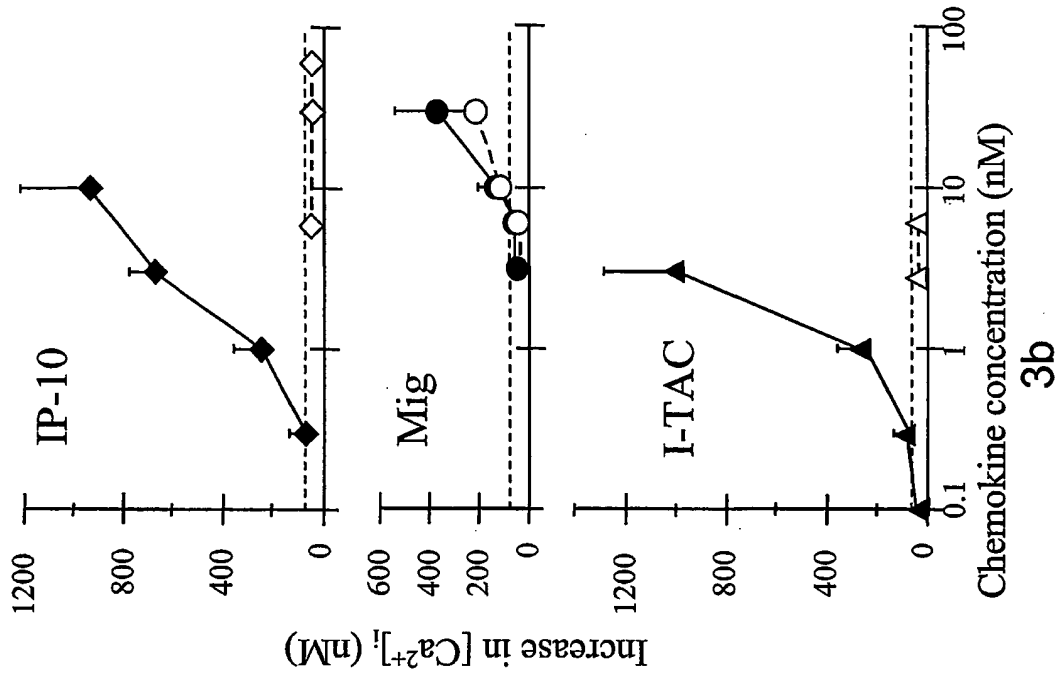
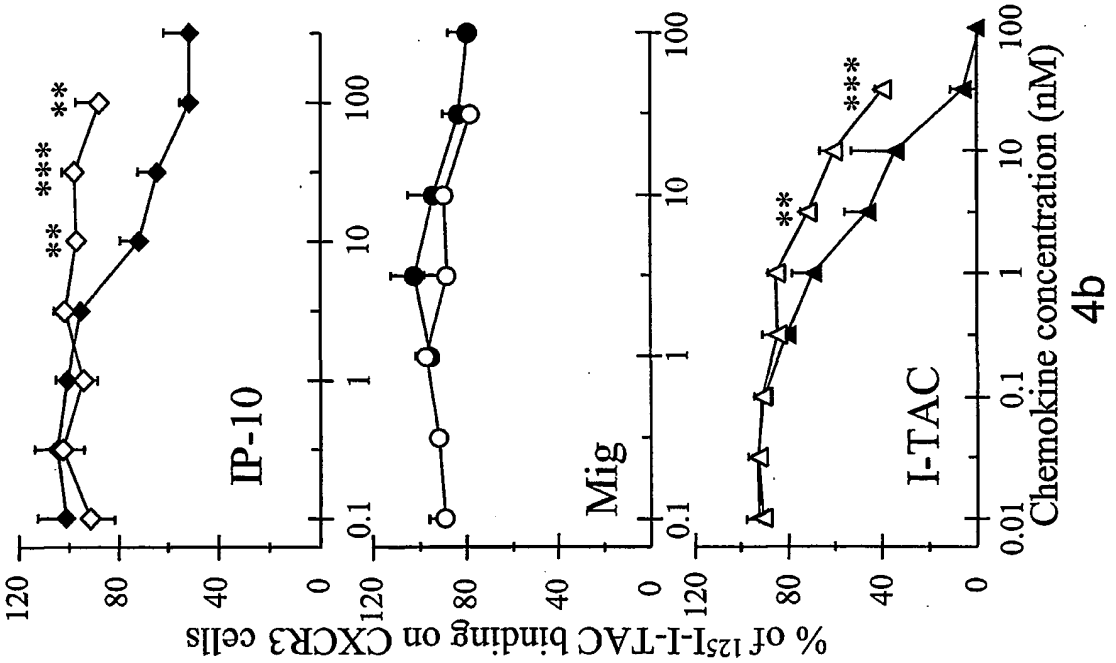
2
3
4
5
6
7
1
3
10
30
**
***
**
***
**
***


IP-10
Mig
I-TAC
Chemotactic index
Chemokine concentration (nM)
3a


IP-10
Mig
I-TAC
Increase in [Ca2+]i (nM)
Chemokine concentration (nM)
3b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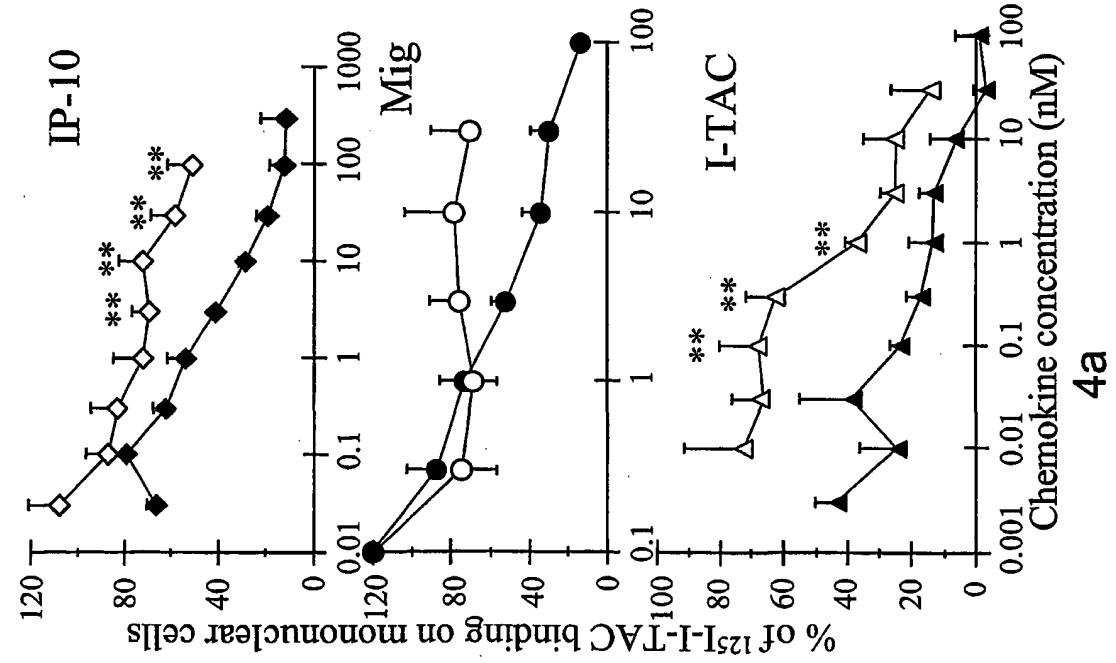
IP-10
Mig
I-TAC
Chemokine concentration (nM)
4b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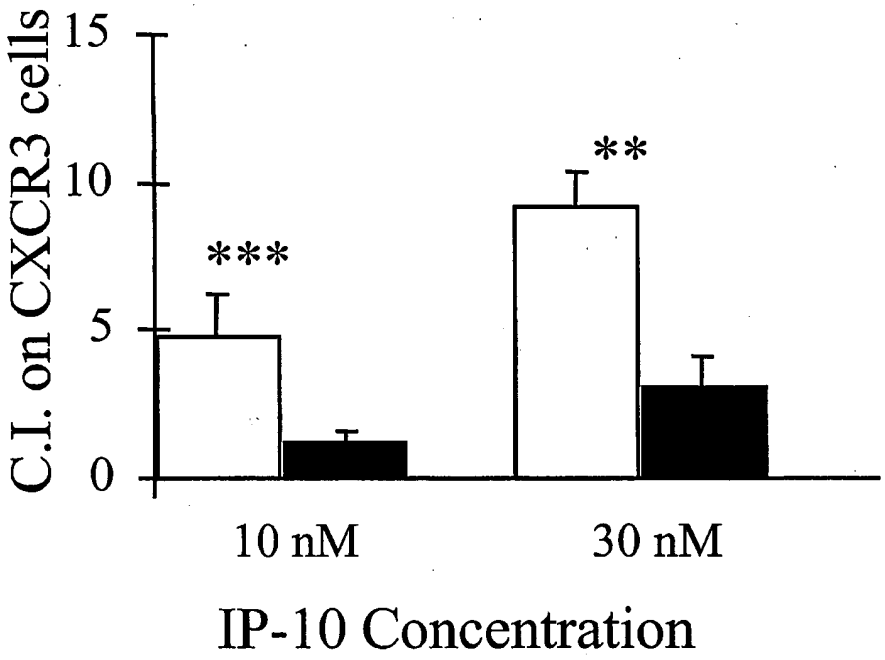
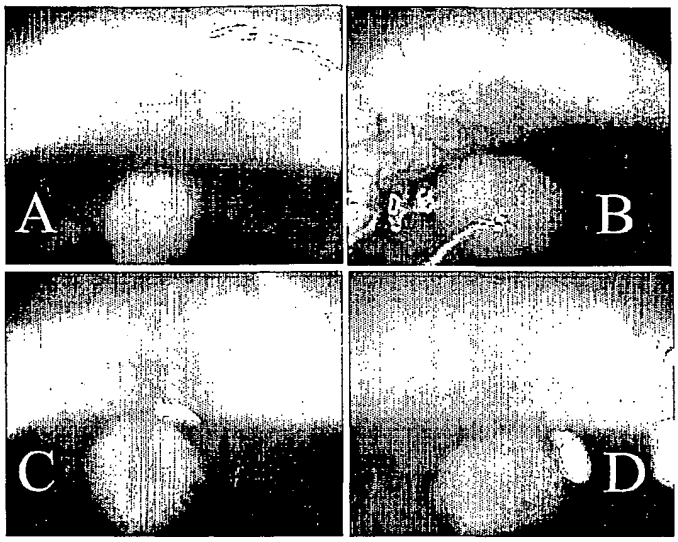
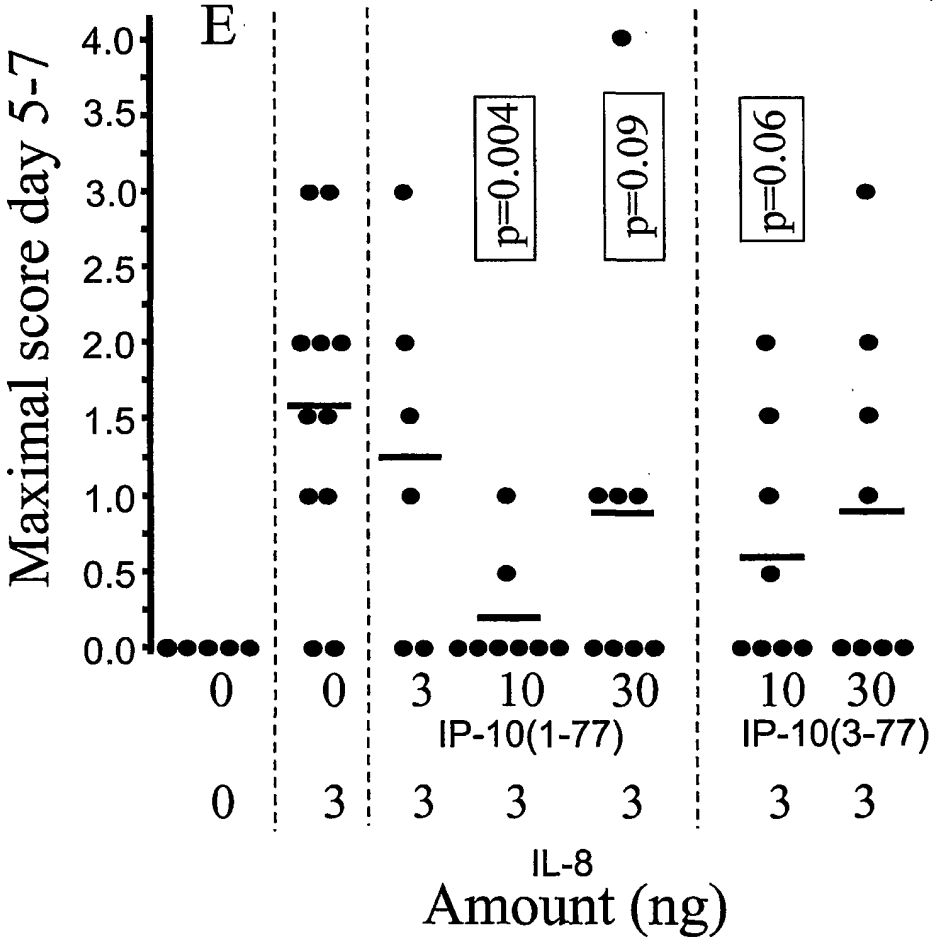
Mig
I-TAC
Chemokine concentration (nM)
4a


C.I. on CXCR3 cells
15
10
5
0
***
**
10 nM
30 nM
IP-10 Concentration


A
B
C
D


E
Maximal score day 5-7
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3.5
3.0
2.5
2.0
1.5
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0
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3
10
30
10
30
IP-10(1-77)
IP-10(3-77)
0
3
3
3
3
3
3
IL-8
Amount (ng)

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 02/00011

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/52 C12Q1/68 C12N5/10 G01N33/68
A61K38/17 //A61P17/00,A61P17/00,A61P19/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | ORAVECZ TAMAS ET AL: "Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 186, no. 11, 1 December 1997 (1997-12-01), pages 1865-1872, XP002197945 ISSN: 0022-1007 page 1866, paragraph 2 --- | 1-7 |
| X | WO 99 28474 A (ORAVECZ TAMAS ;US HEALTH (US); NORCROSS MICHAEL A (US)) 10 June 1999 (1999-06-10) page 23, paragraph 2 --- -/-- | 1-7 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | VAN DAMME JO ET AL: "The role of CD26/DPP IV in chemokine processing." CHEMICAL IMMUNOLOGY, vol. 72, 1999, pages 42-56, XP001073657 1999 S. Karger AG; S. Karger AG P.O. Box, Allschwilerstrasse 10, CH-4009 Basel, Switzerland; New York, New York, USA ISBN: 3-8055-6861-4 table 1 --- | 1-7 |
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1, 2, 4-6 relate to a method defined by reference to a desirable characteristic or property, namely the ability to truncate a chemokine by treament with a protease resulting in a truncated chemokine with abrogated CXCR signalling, but retained antiangiogenic activity. The claims cover all methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT only for treatment with dipeptidyl peptidase IV. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the methods comprising treatment with dipeptidyl peptidase IV.

Present claims 7-13 relate to an extremely large number of possible chemokines. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the chemokines claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the chemokines being substrates for CXCR3 namely, Mig, IP10 and I-Tac.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 02/00011

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